Isolation of five carotenoid compounds from tangerine tomatoes

Thesis

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I. Abstract

Tomatoes are widely consumed in the American diet, and epidemiological data suggest health benefits from consumption. It is hypothesized that these observed health benefits may be caused by carotenoids, a class of pigments widely found in fruits and vegetables. Well-known carotenoids include beta-carotene and lycopene. The tangerine tomato is an orange colored tomato; its color difference can be attributed to a different carotenoid profile compared to red tomatoes. Some carotenoids present in tangerine tomatoes are suggested to be more bioavailable. The goal of this project was to isolate and purify five carotenoids from tangerine tomatoes to use as authentic standards, as these compounds are not readily available for purchase so they must be prepared in the lab immediately before use. To be able to assess bioavailability or bioefficacy, these carotenoids must be quantified in animal and/or human blood and tissues after ingestion of tangerine tomatoes. Authentic standards of each compound must be available in order to determine the amount present in the biological samples. Phytoene, phytofluene, zeta-carotene, neurosporene, and tetra-cis lycopene (also known as prolycopene) were extracted from tangerine tomatoes using nonpolar solvents. These compounds were separated and fractionated using preparative high performance liquid chromatography with photodiode array detection (HPLC-PDA). The purity of these fractions was then assessed using analytical HPLC-PDA. These five purified carotenoid standards were used to prepare calibration curves for assessing bioavailability differences between red and tangerine tomatoes in humans. Additionally, these standards will be used to quantify carotenoids in serum and tissues from other pre-clinical and clinical studies involving red

and tangerine tomato diets. Isolating these carotenoid compounds is essential step in continuing this research on the effects of tangerine tomato consumption on health.

II. Introduction

i. Objective

The main objective of this research is to isolate five carotenoid compounds from tangerine tomatoes using preparative high performance liquid chromatography (HPLC). These compounds need to be isolated so that they can be used as analytical standards for other studies being completed in the Schwartz lab.

ii. Previous studies

Previous studies that relate to this work include chromatographic methods used to separate carotenoids from tomatoes and biological samples. These studies were the basis of the methods used in this research but they were highly modified in order to obtain the desired results. The background research involved in this project included learning about HPLC theory and training on the equipment. See the methods section for details on the chromatographic methods used.

iii. Background

High performance liquid chromatography is a laboratory technique used to separate compounds in a sample. Separation is achieved by passing the sample through a column in which some analytes interact more strongly with the column than others; thus these

analytes have different “retention times” or amount of time before they pass through the column. The column used in HPLC can separate compounds from a sample based on structure, hydrophobicity, ionic charge, ligand affinity, or other means. This research used C30 and C18 columns which interact with analytes based on hydrophobicity. HPLC has an advantage to traditional column chromatography because the system is under high pressure which allows columns to be tightly packed with narrow diameters. This allows for separation of compounds very similar in structure and polarity (such as the target compounds in this research).

After separation in the column the compounds are analyzed using a detector which varies based on the application. A common detector used in conjunction with HPLC is photodiode array detector (PDA) which emits light in the ultraviolet through the visible range (roughly 200-700 nm). This light passes through the analytes as they exit the column and the absorbances of the analytes is shown on a computer attached to the machine. Carotenoids are colored compounds which are found in a multitude of fruits and vegetables. Individual carotenoids can be distinguished based on their absorbance spectra as they absorb light in the UV-vis range.

iv. Problem identification and justification

The following five compounds need to be separated using preparative high performance liquid chromatography: tetra-cis lycopene, neurosporene, zeta-carotene, phytoene, and phytofluene. These compounds are very similar in structure and polarity which makes them difficult to separate and isolate. Much of the work that was done during the course of this research was modifying HPLC methods in order to actually achieve

separation between the compounds. The similar polarity and structure made this separation difficult, especially considering that this was done on the preparative scale versus the analytical scale. The difference between analytical and preparative is that the purpose of analytical HPLC is to identify and quantify the target analyte(s); whereas the purpose of preparative HPLC is to isolate and purify the target analyte(s) for further use. Analytical HPLC columns have a much smaller column width than preparative columns, and this facilitates better analyte separation and resolution.

These compounds must be isolated in our lab because they are unavailable for purchase from chemical suppliers. Some are unavailable because they would degrade during shipping and processing and/or are extremely expensive, while others are simply not sold by chemical suppliers.

The justification for isolating these compounds is because they are needed for use as analytical standards. Analytical standards are pure compounds which are used in HPLC-PDA and HPLC-MS in order to quantify these compounds in biological samples. Specifically, a Ph.D candidate in our lab was doing a mouse study involving consumption of tangerine tomatoes and skin cancer. After the mice are sacrificed, this researcher will quantify levels of these five compounds in the mice tissue samples. In order to quantify these compounds with HPLC-PDA/MS, the researcher needs to first run the analytical standards and create a calibration curve. The reason for quantifying these analytes in biological samples is to examine any possible correlation between the levels of carotenoids in mouse tissue versus the incidence of tumor growth or other biomarkers of intensive skin damage.

III. Methods

i. Tangerine tomato cultivation

The tangerine tomatoes used for this research was grown at the tomato breeding and genetics lab by Dr. David Francis at Ohio Agricultural Research and Development Center (OARDC). A portion of these tomatoes were processed into juice using a hot break process; this juice was then dried into a powder and stored at -20° C until needed.

ii. Carotenoid extraction

A nonpolar solvent extraction method was used to selectively extract the carotenoid compounds from dried tangerine tomato powder; this is an unpublished method used in the Schwartz lab. About 0.5 grams of tangerine tomato powder were crushed and ground into powder, then 5 mL methanol was added to the powder and vortexed. This mixture is sonicated for 9 seconds then centrifuged at 5,000 RPM for 5 minutes. Next 5 mL of 1:1 acetone:hexane are added to the solution, vortexed, sonicated and centrifuged. The top hexane layer is pipetted off; this layer will be orange/yellow as it contains the carotenoid compounds. The carotenoid containing hexane solution is dried down under nitrogen gas. This extract is then used in the HPLC methods below.

iii. HPLC methodology

There were two different HPLC methods used during this research: a C18 preparative method (adopted and heavily modified from the Isaacson[1] et al method) and a C30 preparative method (adopted and modified from the C30 method used in the Schwartz lab[2]). These methods were initially intended for use on the analytical scale thus they had to

be modified for use on the preparative scale. The injection volume for the preparative scale was determined using the following equation:

$$Vol_{prep} = Vol_{Analytical} * \frac{D^2{}_{Prep}}{D^2{}_{Analytical}} * \frac{L_{Prep}}{L_{Analytical}}$$

Where D is the inner diameter of each column (mm) and L is the length of the column (mm) and $Vol_{analytical}$ is the injection volume on the analytical scale. Using this equation resulted in a preparative injection volume of 100 µL. Next the flow rate for the preparative scale was calculated using the equation:

$$Flow_{prep} = Flow_{Analytical} * \frac{D^2{}_{Prep}}{D^2{}_{Analytical}}$$

Using this equation resulted in an analytical flow rate of 22 mL/minute, which was not possible with the equipment used in the research. So a lower flow rate of 15 mL/min was used for both methods.

The analytical methods did not translate well to the preparative column thus the solvent flow gradient had to be significantly modified as well. See Table 1 for the final C18 method after modification and optimization. After injection and separation, the eluate was collected manually by attaching a 6 inch section of HPLC tubing to the "out" port of the PDA and collecting in glass vials by hand.

A Sunfire Preparative C18 column (19 x 150 mm) was used for the C18 methods and a YMC Preparative C30 column (19 x 200 mm) was used for the C18 methods.

IV. Results and discussion

All five compounds were successfully isolated to an acceptable level for use as analytical standards. These isolated compounds were used to create calibration curves for each respective compounds for continued research in our lab. The isolation of these five compounds was handled on a case-by-case basis. For example: phytoene could be isolated to an acceptable purity after one injection with the C18 method (after extensive optimization of the method). However tetra-cis lycopene and neurosporene were much more difficult to separate from each other and from other trace cis-lycopene isomers. These compounds had to be run through the HPLC column several times in order to reach an acceptable purity level. After a sample was injected, the analytes were collected and run through the column again until an acceptable purity was reached. Sometimes this required use of both the C18 and the C30 column methods.

The methods found in literature had to be significantly modified in order to successfully separate the analytes with the HPLC columns available. The resolution was lower when using preparative C30 and C18 columns versus analytical C30 and C18 columns. Figure 1 shows the chromatogram obtained after injecting a tangerine tomato extract after the initial method modifications. The retention times of the individual compounds were far too close together to collect fractions containing isolated compounds. After changing the solvent gradient and flow rate, the retention times between compounds increased. The peaks obtained using this method were not sharp but they did allow for collection of isolated compounds (Figure 2), which was the objective of the research. Phytoene, phytofluene and zeta-carotene were collected using only the C18 method (see

Figure 2). See Figure 3 for a comparison of the absorbance spectra of these compounds versus literature reference examples. As mentioned before, these compounds were purified in order to be used as analytical standards to quantify them in biological samples. See Figure 4 for the phytoene, phytofluene and zeta-carotene calibration curve.

V. Conclusions and future work

Phytoene, phytofluene, zeta-carotene, neurosporene, and tetra-cis lycopene were successfully isolated from tangerine tomatoes using preparative high performance liquid chromatography. This was completed using a mixture of C18 and C30 chromatographic methods as needed for each compound. These compounds were used in our lab to create calibration curves for quantification of the compounds in biological samples.

VI. Acknowledgements

I would like to thank my research advisor, Dr. Steven Schwartz, for allowing me to work in his lab and gain invaluable experience along the way. Additionally, I would like to thank Jessica Cooperstone who guided me along the research process and trained me in all of the lab processes. All of the research done in this experiment used equipment and supplies available in the Schwartz Lab. These supplies are funded by combination of research grants and the Carl E. Haas endowment.

VII. Bibliography

[1]Isaacson T, et. Al. 2002. Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of beta-carotene and xanthophylls in plants. Plant Cell. 14(2):333-342

[2]Ferruzzi M, Schwartz S, et al. 1998. Carotenoid Determination in Biological Microsamples Using Liquid Chromatography with a Coulometric Electrochemical Array Detector. Anal. Biochem. 256:74-81.

[3]Britton G, Liaaen-Jenson S, Pfander H. Carotenoids: Handbook. 2004; 1st edition. Birkhauser, Basel, Switzerland.

VIII. Supplementary material

Table 1. Modified C18 preparative HPLC method used to separate carotenoids in tangerine tomatoes. Solvent A: 90% acetonitrile, 8% H_2O, 2% of 2% ammonium acetate solution (aqueous). Solvent B: 100% ethyl acetate.

Time	% A	% B
0	65	35
13	50	50
20	45	55
22	0	100
22.01	65	35
26	65	35

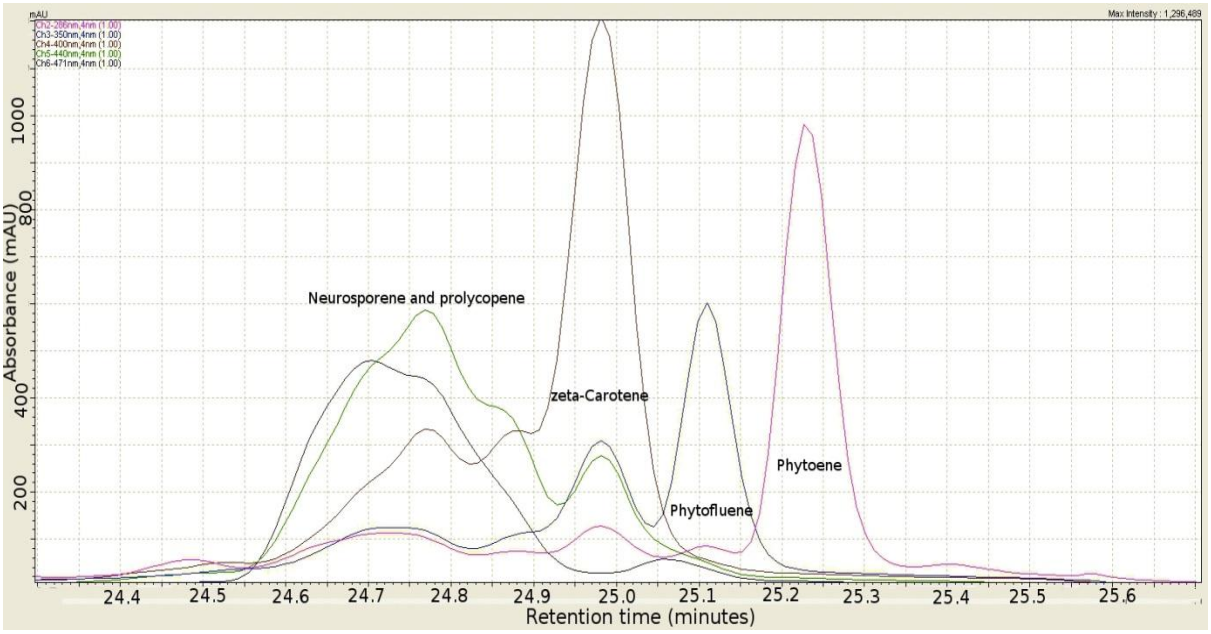

Figure 1. Chromatogram for C18 prep-HPLC run before modification. Note that all carotenoids eluted in under 60 seconds; the compounds could not be isolated when the retention times were so similar to each other

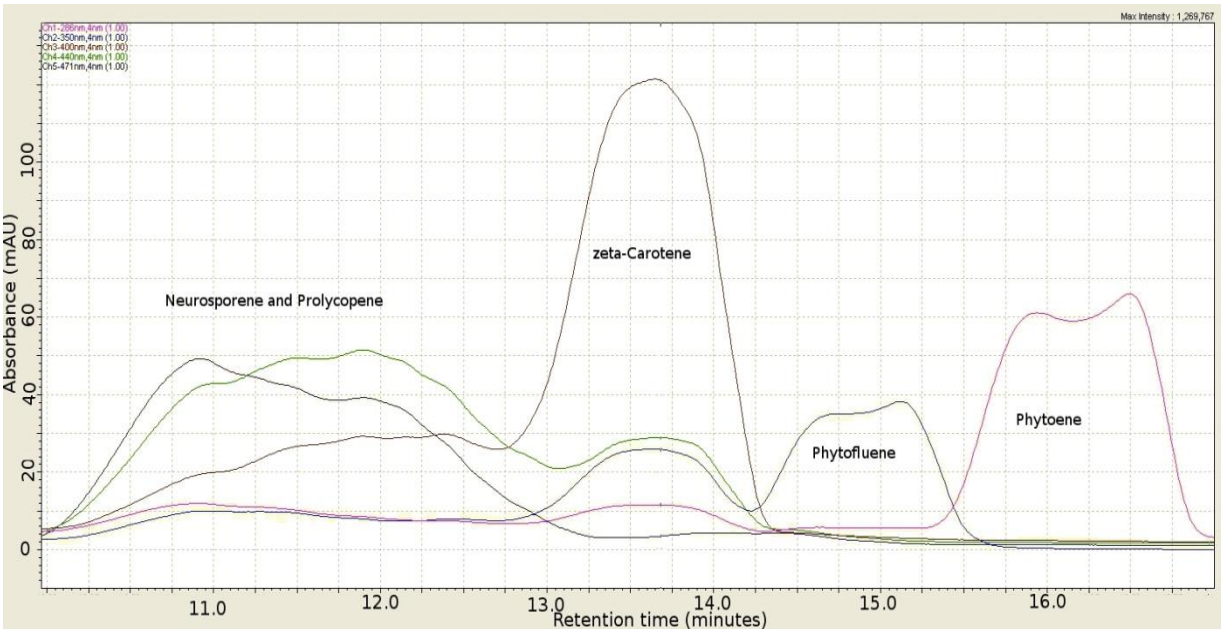

Figure 2. Chromatogram for C18 prep-HPLC run after method modification. Phytoene, phytofluene and ζ-carotene have retention times which are more spread out thus they could be effectively isolated by collecting the eluate off the column

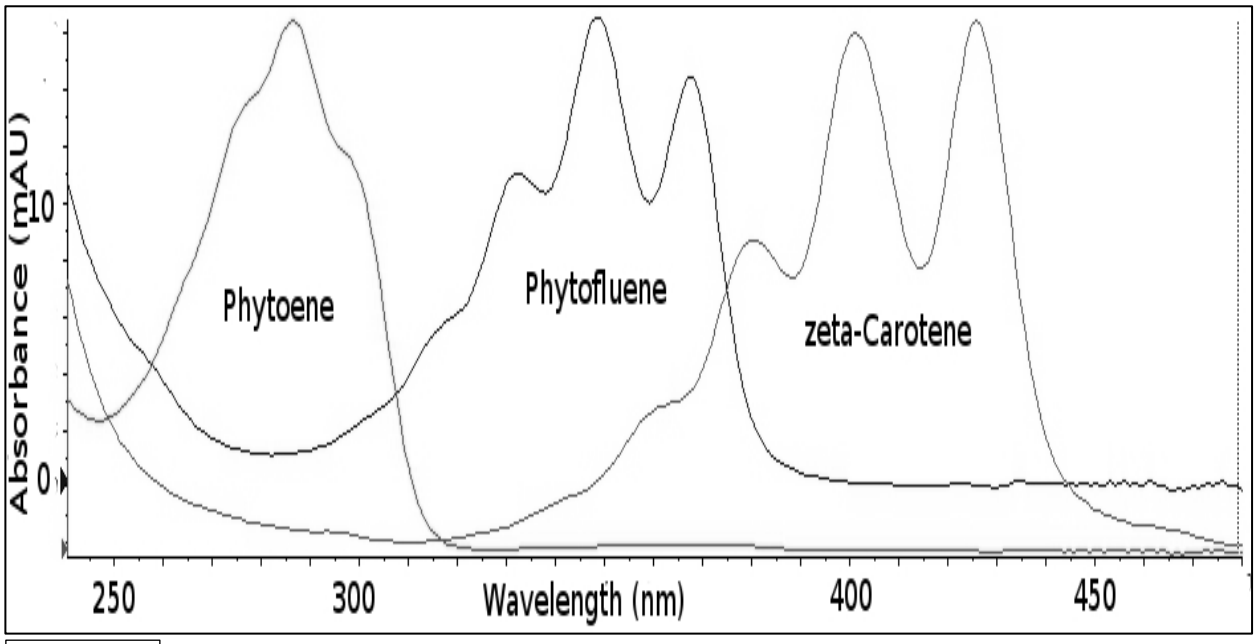

Figure 3a

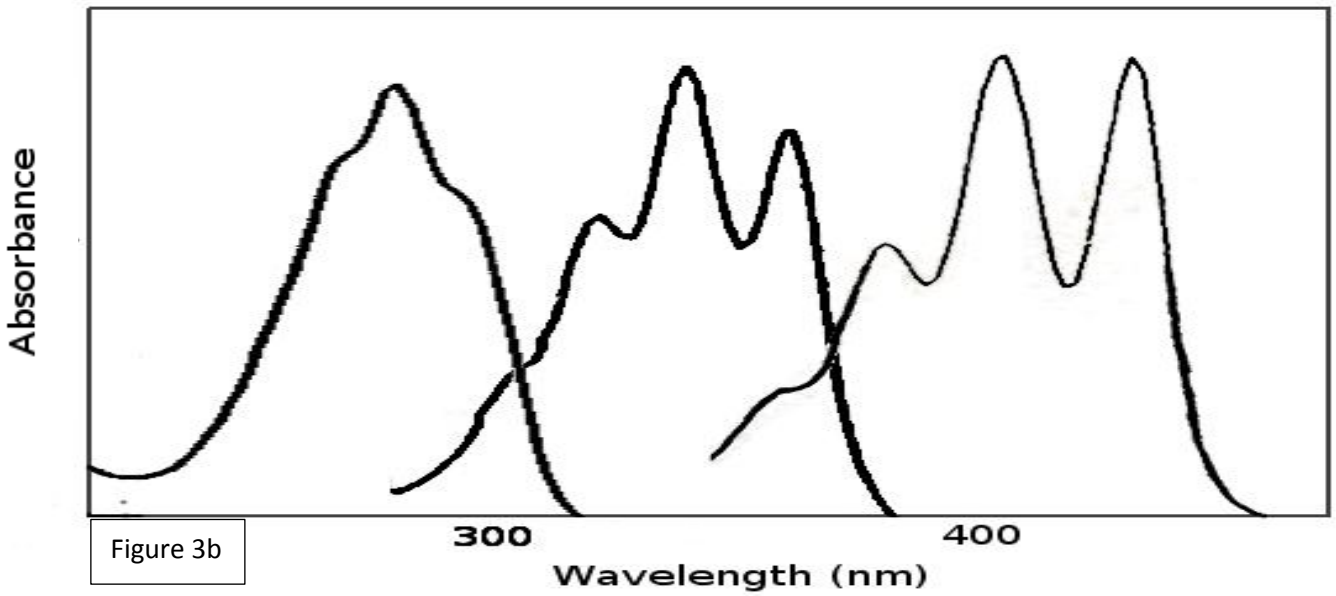

Figure 3b

Figure 3: Comparison of experimental spectra obtained after purification of phytoene, phytofluene and zeta-carotene. Figure 3a shows experimental spectra collected during the course of this research while Figure 3b shows reference spectra from a carotenoids reference handbook[3]

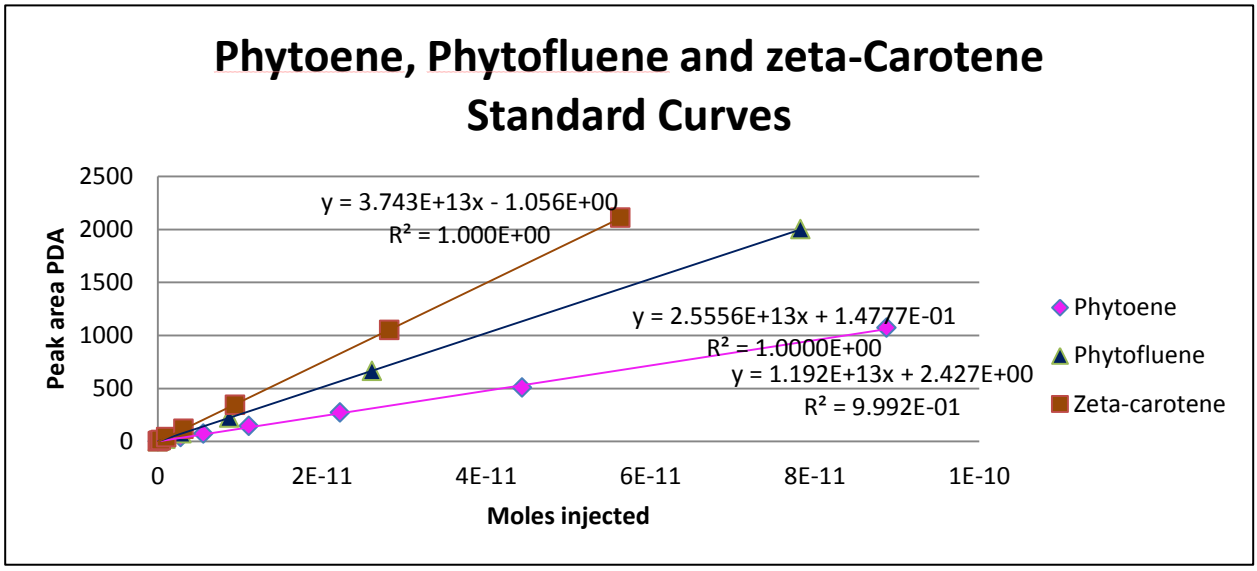

Figure 4. Calibration curves created from three of the five compounds isolated during this research (phytoene, phytofluene and zeta-carotene)